Journal of Medical Virology 58:165–173 (1999)

# Unintegrated HIV-1 Circular 2-LTR Proviral DNA as a Marker of Recently Infected Cells: Relative Effect of Recombinant CD4, Zidovudine, and Saquinavir In Vitro

**Lori A. Panther, Robert W. Coombs,* Sandra A. Aung, Corazone dela Rosa, David Gretch, and Lawrence Corey**

*Departments of Laboratory Medicine and Medicine, University of Washington, Seattle, Washington*

Unintegrated HIV-1 proviral DNA is one of the earliest detectable forms of HIV-1, and the influence of an antiretroviral drug on its appearance may reflect the efficacy of that agent in preventing infection of new cells. We characterized the dynamics of HIV-1 p24 (p24) antigen production, HIV-1 *gag* DNA, tandem long-terminal-repeat circular unintegrated proviral (2-LTR) HIV-1 DNA, HIV-1 *tat* mRNA, and cell viability in the presence of three antiretroviral agents: recombinant soluble CD4 (rsCD4), zidovudine, and saquinavir. Interference with HIV-1 entry by rsCD4 decreased p24 antigen levels modestly, decreased HIV-1 *gag* by twofold, and 2-LTR was detectable at the end of the culture period. Inhibition of reverse transcription by zidovudine decreased p24 antigen levels modestly, decreased HIV-1 *gag* by 19-fold, and inhibited detection of 2-LTR HIV-1 DNA. The protease inhibitor, saquinavir, had the greatest overall effect, with the lowest levels of p24 antigen and HIV-1 *gag,* and inhibition of 2-LTR. There was no detection of *tat* mRNA in the saquinavir-treated cultures. In addition, cell viability was significantly higher in cultures treated with saquinavir. In these experiments, 2-LTR HIV-1 DNA was indicative of the relative inhibitory effects of three antiretroviral agents acting at different steps of the HIV-1 replication cycle. We demonstrated in vitro that 2-LTR HIV-1 DNA was a useful indicator of an antiretroviral drug in preventing new cell infection and could be utilized as a dynamic marker of drug efficacy in HIV–1–infected patients. ***J. Med. Virol. 58:165–173, 1999.*** 

**KEY WORDS:** HIV-1 entry; reverse transcriptase; protease inhibitor; HIV-1 *tat* mRNA; tandem long terminal repeat; antiretroviral

## INTRODUCTION

Multiple copies of unintegrated proviral DNA per cell have been demonstrated in tissue culture infections with cytopathic animal retroviruses. Examples include equine infectious anemia virus [Rasty et al., 1990], visna virus [Brahic et al., 1981], feline leukemia virus [Mullins et al., 1986], spleen necrosis virus [Chen and Temin, 1982], avian leukosis virus [Weller and Temin, 1981], and reticuloendotheliosis virus [Temin et al., 1980]. Similarly, unintegrated human immunodeficiency virus type-1 (HIV-1) proviral DNA has been demonstrated in acute in vitro infections of lymphocytes [Pauza et al., 1990; Robinson and Zinkus, 1990], monocytes [Besansky et al., 1991], and chronically infected cell lines [Chowdhury et al., 1992]. Moreover, unintegrated HIV-1 provirus is inducible upon stimulation of these chronically infected cells. High amounts of unintegrated HIV-1 DNA found in mononuclear cells and tissues of HIV-infected patients is associated with clinical progression of HIV disease [Levy et al., 1985; Shaw et al., 1985; Pang et al., 1990; Zazzi et al., 1997; Panther et al., 1998]. Recent studies have shown decreases in unintegrated HIV-1 DNA [Dickover et al., 1992; Bush et al., 1993; Donovan et al., 1994a, 1994b; Pauza et al., 1994; Nicholson et al., 1996] in the peripheral blood mononuclear cells (PBMCs) of patients after starting antiretroviral monotherapy, suggesting that unintegrated HIV-1 DNA is a dynamic marker of infection of new cells. Consequently, unintegrated

Lori A. Panther's current address is Department of Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts.

Grant sponsor: National Institutes of Health; Grant numbers: NS-25183, AI-27764, AI-27757, and AI-30731.

*Correspondence to: Dr. Robert W. Coombs, Department of Laboratory Medicine, Room NW-120, Box 357110, University of Washington, Seattle, WA 98195. E-mail: bcoombs@u.washington.edu

Accepted 4 November 1998

HIV-1 DNA may also be useful to understand the relative efficacy of different antiretroviral agents in vitro and in vivo [Donovan et al., 1994a, 1994b; Bush et al., 1996; Zazzi et al., 1997].

The exact mechanism responsible for the intracellular accumulation of unintegrated proviral DNA is unknown but either reinfection of cells or cell-to-cell transfer of viral DNA [Pauza et al., 1990; Sato et al., 1992; Laurent-Crawford and Hovanessian, 1993; Sonza et al., 1994; Guan et al., 1997; Marquina et al., 1997] seem most plausible. In acutely infected cells, accumulation of unintegrated HIV-1 DNA may result from failure to establish viral interference [Stevenson et al., 1988; Kawamura et al., 1989]. In chronically infected cells, multiple copies of unintegrated HIV-1 DNA may result from entry of multiple virions via reestablished surface receptors, syncytium formation, or alternate mechanisms of viral entry [Temin, 1988; Guan et al., 1997]. Understanding these mechanisms is important for explaining the persistence of unintegrated HIV-1 proviral DNA after potent antiretroviral therapy [Chun et al., 1997a, 1997b].

Unintegrated HIV-1 DNA assumes several monomeric and multimeric forms [Farnet and Haseltine, 1991]. We used polymerase chain reaction (PCR) amplification to detect the circular 2-LTR form of HIV-1 DNA (2-LTR) that results from end-to-end joining of the provirus. As such, 2-LTR is a specific marker of a completely reverse-transcribed, unintegrated infectious viral RNA genome [Kulkosky et al., 1990; Farnet and Haseltine, 1991]. Our goal was to characterize the dynamics of 2-LTR in an in vitro cell culture system by using a syncytium-inducing HIV-1 isolate to study the effects of three antiretroviral agents that act at different points in the viral replication cycle. We used recombinant soluble CD4 (rsCD4), which blocks entry of the virus into $CD4^+$ cells, zidovudine, which inhibits HIV-1 reverse transcription, and saquinavir, a protease inhibitor known to interfere with posttranscriptional processing of virions and that may also inhibit replication steps prior to integration [Nagy et al., 1994].

## MATERIALS AND METHODS

### Cells and Virus Strain

CEM and ACH-2 cells were maintained at a density of $1 \times 10^5$ to $1 \times 10^6$/mL in RPMI 1640 supplemented with 10% fetal bovine serum, penicillin, streptomycin, DEA-dextran, and L-glutamine [Foley et al., 1965]. The cells were passaged 48 hr prior to infection. In two experiments, HIV-1–negative human peripheral blood mononuclear cells maintained at a density of $1 \times 10^5$ to $1 \times 10^6$/mL in RPMI 1640 supplemented with 10% fetal bovine serum, penicillin, streptomycin, DEA-dextran, L-glutamine, and phytohemagglutinin (PHA) were used [Coombs et al., 1989]. For all experiments, we used aliquots of cell-free $HIV_{LAI-1}$ virus that had been titered by an endpoint dilution assay on CEM host cells and stored at −70°C.

### Antiretroviral Drugs

$HIV_{LAI-1}$ was cultured in CEM cells in the presence of either 200-nM rsCD4 (Biogen, Boston, MA), 1-μM zidovudine (Glaxo-Wellcome, Research Triangle Park, NC), or 30-nM saquinavir (Hoffman-La Roche, Welwyn Garden City, Hertfordshire, England). The dosages of these agents were equal to or exceeded the IC90 in other in vitro studies of inhibition of $HIV_{LAI-1}$ infection in T-cell lines [Gomatos et al., 1990; Craig et al., 1991; Masquelier et al., 1991]. $HIV_{LAI-1}$ was cultured in PBMCs in the presence of either zidovudine or protease inhibitor at the above concentrations.

### Experimental Design

Cells ($50 \times 10^6$) were incubated with 8,000 tissue culture infectious dose 50% (TCID50) of HIV-1 for 1 hr at 37°C in a total volume of 10 mL. The cells were then washed four times with 30 mL of phosphate-buffered saline (PBS) and pelleted after each wash in a Beckman model TJ-6 centrifuge at 1,200 × g for 10 min at 4°C. In six cultures, an aliquot of the fourth PBS wash-supernatant was assayed for HIV-1 p24 antigen to ensure that cell-free virus was effectively removed and also to establish a baseline for HIV-1 p24 antigen measurements. The cells were then resuspended in PBS, counted by trypan blue exclusion, and aliquots of $5 \times 10^6$ cells were placed into four culture flasks designated for harvesting on days 2, 3, 4, or 7. The cells and virus were cultured in the presence of rsCD4, zidovudine, or protease inhibitor. In all experiments, virus controls were cultured in the absence of drug.

One flask from each of the control and drug-treated cultures was harvested on days 2, 3, 4, and 7. Culture supernatants were saved for HIV-1 p24 antigen measurement. Cells were pelleted by centrifugation, washed twice, resuspended in PBS, counted, and viability was assessed by trypan blue exclusion. Aliquots of $5 \times 10^4$ cells from each flask were added to 1.5-mL Eppendorf vial containing $2 \times 10^6$ uninfected carrier PBMC and set aside for DNA extraction and polymerase chain reaction (PCR) amplification. From each of the CEM cultures treated with protease inhibitor, an aliquot of cells at each harvest was added to 500-μL activated guanidinium thiocyanate (GuSCN) for *tat* mRNA analysis.

### HIV-1 p24 Antigen

Aliquots of culture supernatant were taken from each culture on days 2, 3, 4, and 7 for p24 antigen quantitation (Abbott Laboratories, Abbott Park, IL). For HIV-1 p24 antigen levels above 600 pg/mL, the supernatants were diluted 10-fold and reassayed for HIV-1 p24 antigen.

### HIV-1 Proviral DNA Preparation

Each aliquot of $5 \times 10^4$ cells saved for PCR was resuspended in 167 microliters each of: a buffer solution comprised of 100-mM KCl, 10-mM Tris-HCl (pH 8.3), and 2.5-mM $MgCl_2$; a detergent solution comprised of 10-mM Tris-HCl (pH 8.3), 2.5-mM $MgCl_2$, 1% Tween

20, 1% Nonidet P-40, and 0.12-mg proteinase-K/mL, to give a final concentration of $6 \times 10^6$ cells/mL, of which there were 7,500 treated cells per PCR amplification reaction. The samples were incubated in a 60°C water bath for 1 hr and then placed in a 95°C heating block for 10 min to inactivate the proteinase K.

### HIV-1 RNA Preparation

To each aliquot of $5 \times 10^4$ cells in GuSCN saved from the protease inhibitor cultures was added 50 μL of 2M sodium acetate (pH = 4), 500-μL water-saturated phenol, and 100 μL of 49:1 chloroform/isoamyl alcohol. This mixture was incubated 15 min on ice and centrifuged for 20 min at 12,000 rpm (4°C). To the top aqueous phase was added one volume of isopropanol and the sample was incubated for 30 min at –20°C, then centrifuged 20 min at 14,000 rpm (4°C). The resulting RNA pellet was dissolved in 250-μL GuSCN and precipitated again with 250-μL isopropanol. The pellet was washed with 75% ethanol, dried, and dissolved in 10-μL DEPC-treated water. Reverse transcription of RNA to cDNA was done by adding to each sample 100 pmol of random hexamer primers (Pharmacia, Piscataway, NJ), 10-U Moloney murine leukemia virus (M-MLV) reverse transcriptase (Boehringer Mannheim, Indianapolis, IN), 1 mM of each dNTP, and 25 units of RNAsin in a buffer of 50-mM KCl, 20-mM Tris-HCl (pH 8.4), 2.5-mM $MgCl_2$, and 100-mg/mL nuclease-free bovine serum albumin, for a final volume of 25 μL. The samples were incubated 1 hr at 37°C and then for 5 min at 95°C to inactivate the enzymes.

### HIV-1 DNA PCR

Proviral HIV-1 DNA PCR amplifications were performed separately to detect total HIV-1 *gag* and 2-LTR unintegrated HIV-1 DNA. To 50 μL of a sample DNA preparation were added 50 μL of master mix such that final reaction conditions were 50-mM KCl, 10-mM Tris (pH 8.4), 2.5-mM $MgCl_2$, 0.01% gelatin, 0.2 mM each of dATP, dCTP, dGTP, and dTTP, and 2.5-U Taq polymerase (U.S. Biochemical, Cleveland, OH) in a total volume of 100 μL. Primers SK38 and SK39 [Kwok et al., 1988] were used to amplify a portion of the HIV-1 *gag* gene, and primers U3 and U5 were used to amplify the 2-LTR circular HIV DNA [Whitcomb et al., 1990]. The samples were placed in a Perkin-Elmer Cetus DNA Thermal Cycler and the PCR reaction was performed using a step-cycle file programmed for 30 cycles of 89°C for 40 sec and 62°C for 80 sec.

The *gag* and 2-LTR PCR products were labeled using a liquid hybridization procedure with nested probes end-labeled with ($\gamma$-$^{32}$P). The probe SK19 [Ou et al., 1988] was used to detect the 113-bp *gag* PCR product. A nested oligonucleotide probe ("P2" 5′-GGAAAATCTCTAGCAGTACTGGAAGGGC-TAATTCA-3′) spanning the 2-LTR circle junction sequence was designed to detect the 132-bp 2-LTR product. $^{32}$P-labeled PCR products were detected by electrophoresis on a 10% polyacrylamide gel and autoradiography. Semiquantitation of the PCR products was done by comparing band intensities with a standard curve included in each PCR run; copy number was estimated as either equal to or exactly in between two dilutions of the standard curve depending on band intensity. PCR standard curves for $HIV_{LAI-1}$ DNA were derived from 10-fold dilutions of ACH-2 cells, which contain one integrated copy of the HIV-1 genome per cell. Standard curves for 2-LTR were derived from 10-fold dilutions of a pTZ19U plasmid into which had been subcloned a 590-bp DNA sequence containing the 2-LTR circle junction [Smith et al., 1990]. Junction phage was diluted in 50 ng/μL of human placental DNA to a negative endpoint. Using these standards, the reproducible sensitivity for both *gag* and 2-LTR PCR assays was 10 copies.

### HIV-1 *tat* mRNA PCR

For HIV-1 *tat* mRNA (cDNA) PCR amplification, to each 25-μL cDNA sample was added 2.5-U Taq DNA polymerase (Perkin-Elmer Cetus), 20 pmol each of primers MF5869 and MF8760 [Furtado et al., 1991], 2.25-μL $MgCl_2$, and buffer for an end volume of 50 μL and amplified for 30 cycles. The 550-bp PCR product was detected using liquid hybridization to a $^{32}$P-labeled probe MF5945, electrophoresis on 10% polyacrylamide gel by autoradiography. The intensity of the band at each time point was compared in a relative fashion to the intensity of the control culture at the same time point. Actin primers AT-1 and AT-4 were used as an internal control to assess for fidelity of RNA extraction and reverse transcription.

### Statistical Analysis

Differences among treatment groups were assessed for statistical significance using the one-way ANOVA. When a significant difference among treatment groups was present, multiple comparisons among all pairs of treatment groups were tested using the unpaired *t*-test with Bonferroni correction for level of significance.

## RESULTS

Seven replicate experiments were carried out using 200-nM rsCD4, nine replicates using 1-μM zidovudine, and six replicates using 30-nM saquinavir. Twenty-one control cultures were done in the absence of antiretroviral drug. Two cultures each of PBMCs infected with $HIV_{LAI-1}$ in the presence of either zidovudine or saquinavir were used for comparison of results in different host cell populations.

### Cell Viability

Cell viability was measured in the CEM cell cultures as a general indicator of each drug treatment's protection against viral-induced cell lysis and death in acutely infected cells. Baseline viability of CEM cells was greater than 90%. In control cultures, >90% of the cells were viable at the time of infection and remained so until day 4, after which viability decreased substantially (Fig. 1). By day 7, only the saquinavir-treated cultures had significantly higher viability compared to the untreated, infected controls ($P = 0.007$, *t*-test). In

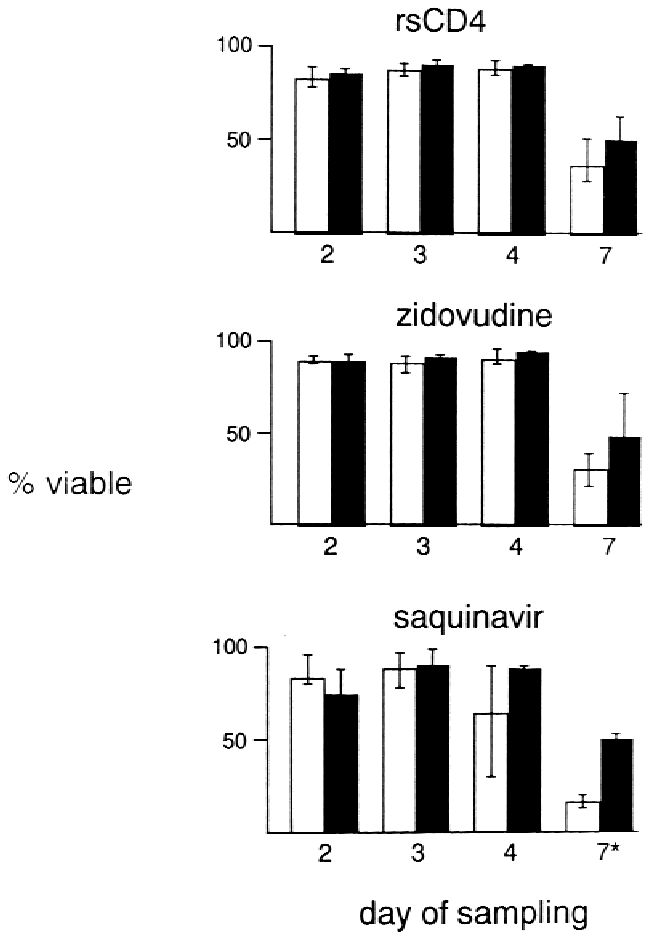


Fig. 1. Cell viability following infection. CEM cell viability was measured by trypan blue exclusion in cells infected with HIV-$1_{LAV-1}$ and cultured in the absence or presence of either rsCD4, zidovudine, or saquinavir. Cell viability at the time of infection was >90%. Seven days following infection, between-treatment group cell viability was not different ($P$ = 0.2, ANOVA). However, within-treatment group cell viability was significantly greater in the saquinavir compared to control treated cultures (*$P$ = 0.007, $t$-test). Bars represent mean values and vertical lines show one standard deviation. Open bars indicate control cultures and solid bars indicate antiretroviral-treated cultures.

the PBMC cultures, viability was similar to that of CEM cells. Cell viability was not affected by the drugs themselves, with >80% of uninfected cells viable after 1 week in the presence of each of the three agents at the concentrations used in the experiments (data not shown).

### HIV-1 p24 Antigen

HIV-1 p24 antigen was measured throughout the culture period to assess the effect of each drug on productive infection in the CEM cells. The mean (SD) HIV-1 p24 antigen level in the supernatant was 3.4 (1.9) pg/mL (n = 6) at the start of the culture period, indicating that subsequent HIV-1 p24 antigen levels represented virus produced from productively infected cells. The HIV-1 p24 antigen levels between treatment group controls were similar throughout the culture period. By the end of the 7-day culture, mean HIV-1 p24 antigen levels were 56-fold lower in rsCD4-treated cultures, 641-fold lower in zidovudine-treated cultures, and 5,210-fold lower in the saquinavir-treated cultures compared to controls (Fig. 2). The HIV-1 p24 antigen levels in the rsCD4- and the zidovudine-treated cultures were not significantly lower than for the controls. However, the HIV-1 p24 antigen level for the saquinavir-treated cultures was significantly lower than for the control at day 7 ($P$ = 0.012, ANOVA). For the PBMC cultures treated with zidovudine and saquinavir, HIV-1 p24 antigen levels in the culture supernatants were similar to CEM cultures treated with these agents (data not shown).

### HIV-1 Proviral DNA PCR

HIV-1 *gag* DNA PCR measured both integrated and unintegrated (linear and circular) reverse-transcribed forms of HIV-1 provirus. In control cultures, HIV-1 *gag* was detected by day 2 and increased throughout the culture period and 2-LTR DNA was detected by days 3 to 4 but at a lower level than HIV-1 *gag* DNA (Figs. 3A and 4). This pattern of band intensity was expected since PCR for *gag* will detect most forms of integrated and unintegrated HIV-1 DNA, whereas PCR for 2-LTR circular HIV-1 specifically detects only one of several forms of unintegrated HIV-1 DNA.

Addition of 200-nM rsCD4 to the culture medium only decreased slightly the PCR *gag* signal intensity compared to untreated controls. Though appearance of the 2-LTR was delayed in the rsCD4-treated cultures, it became consistently detectable by day 4 or 7 and was 10-fold lower compared to controls by day 7. In the cultures treated with 1-μM zidovudine, mean PCR *gag* signal was 19-fold lower compared to controls and 2-LTR was undetectable throughout the culture period. The protease inhibitor, saquinavir, had a marked effect, with a 367-fold reduction in mean *gag* PCR signal by day 7 and undetectable 2-LTR throughout the culture period (Figs. 3A and 4). Breakdown of antiretroviral agents did not explain the PCR findings for either rsCD4 or zidovudine, since daily supplementation of cultures with drug gave similar results compared to cultures that had drug added only at the start of the culture period. PCR findings for PBMC cultures treated with zidovudine or saquinavir showed the superiority of saquinavir to block the production of provirus (Fig. 3B).

To explore further the ability of antiretroviral therapy to block the production of 2-LTR, we stimulated chronically infected ACH-2 cells with phytohemagglutinin (PHA). Treatment with saquinavir (Fig. 5), in contrast to either rsCD4 or zidovudine (data not shown), completely blocked the production of 2-LTR and blunted any rise in HIV-1 *gag* level following PHA stimulation.

### HIV-1 *tat* mRNA PCR

It was of interest to know whether there were undetectable amounts of integrated HIV-1 provirus capable

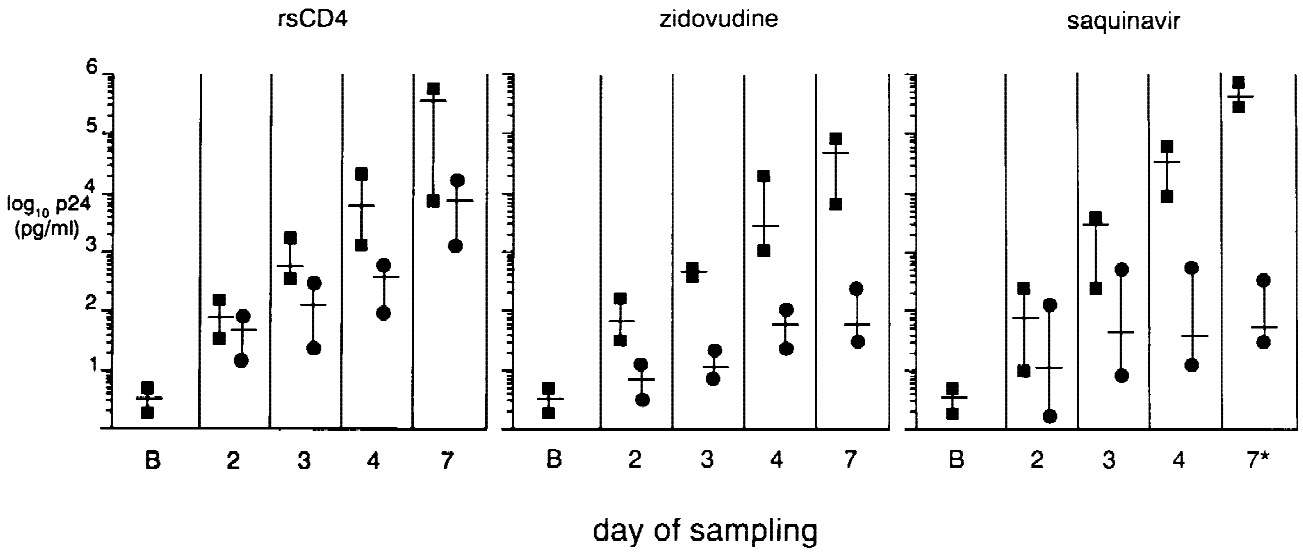


Fig. 2. Effect of rsCD4, zidovudine, and saquinavir on HIV-1 p24 antigen production in HIV-1–infected CEM cells. Vertical bars show interquartile ranges and crossbars show median values. The HIV-1 p24 antigen levels were not significantly different between any of the treatment groups at any time point except between saquinavir and control at day 7 (*$P = 0.012$, ANOVA). Boxes represent infected, untreated control cultures and circles represent drug-treated cultures.

of transcription. To address this question, HIV-1 RNA PCR was performed to detect transcription of the HIV-1 *tat* regulatory gene, which would indicate the presence of low levels of integrated HIV-1 provirus capable of generating a productive infection. In the cultures treated with saquinavir, there was no *tat* mRNA detected, compared to a strong signal produced for *tat* mRNA in infected control cultures (Fig. 6). Actin signal was equal for both drug-treated and control cultures at each time point (data not shown). The consistent detection of *gag* DNA at day 2 of infection (Fig. 3) and *tat* mRNA at day 3 suggested that the *tat* mRNA assay was less sensitive than the *gag* DNA assay for detecting viral replication.

## DISCUSSION

We detected unintegrated 2-LTR circular HIV-1 proviral DNA in cell culture and measured the effect of antiretroviral agents on the accumulation of this proviral DNA moiety in acutely and chronically infected cells. Because 2-LTR circular provirus represents a complete reverse transcription of the viral RNA genome, we postulated that unintegrated 2-LTR circular provirus would be a molecular determinant of infectivity. As such, we showed that this form of provirus was useful for assessing the activity of antiretroviral agents in vitro. This complements our earlier work that showed the in vivo utility of measuring unintegrated 2-LTR circular provirus [Panther et al., 1998].

We found that 200-nM rsCD4 inhibited the accumulation of 2-LTR circular HIV-1 DNA to some extent, but 1-μM zidovudine was more effective in preventing accumulation of circular HIV-1 provirus. Moreover, 30-nM saquinavir prevented accumulation of virtually all HIV-1 reverse transcripts and thus also prevented the formation of 2-LTR circular HIV-1 provirus. The multiplicity of infection used in these experiments was low and there was no PCR signal for HIV-1 *gag* DNA or 2-LTR circular DNA 1 hr after incubation with virus. However, the appearance of high levels of *gag* DNA by day 2 in the control cultures suggested that viral entry into the cells was efficient and that corresponding decreases in *gag* DNA and 2-LTR circular DNA in the presence of antiretroviral agents was most likely explained by the prevention of new cell infection.

High levels of rsCD4 delayed the appearance of *gag* DNA and 2-LTR circular DNA compared to the control cultures, while HIV-1 p24 levels remained relatively high. It is possible that initially infected cells continued to produce HIV-1 that was subsequently bound by rsCD4 in the culture medium. However, the binding capacity of rsCD4 was eventually surpassed and new cell infection was unimpeded by day 7.

Zidovudine (1 μM) totally inhibited the appearance of 2-LTR circular DNA in CEM cells and resulted in a 19-fold decrease in HIV-1 *gag* DNA. Both of these events were associated with a 1,000-fold decrease in HIV-1 p24 antigen by the end of the culture period. Zidovudine's effect on unintegrated 2-LTR circular DNA and *gag* DNA is consistent with inhibition of de novo infection and possibly reinfection of cells by blocking of viral reverse transcriptase.

Saquinavir treatment of the infected cells resulted in extremely low levels of *gag* HIV-1 DNA in both CEM cells and PMBSs and total absence of 2-LTR circular DNA as well as *tat* mRNA. Although HIV-1 p24 antigen levels were substantially lower compared to that for rsCD4- and zidovudine-treated cultures, HIV-1 p24 antigen was still approximately 100 pg/mL by the end of the culture period. It is possible that this low level of

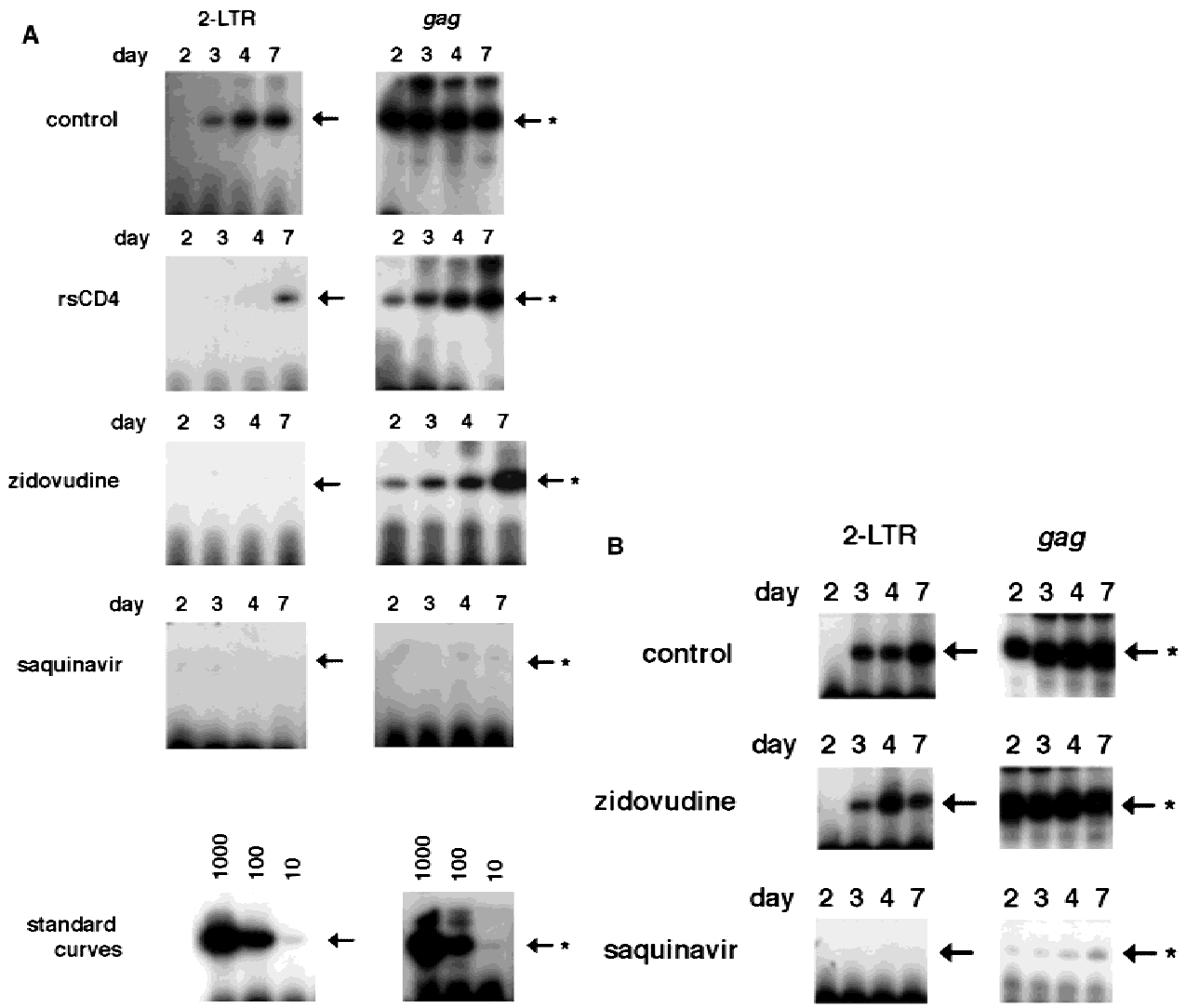


Fig. 3. **A:** Effect of antiretroviral treatment on the appearance of HIV-1 2-LTR and *gag* DNA in CEM cells. PCR products were detected using $^{32}$P-labeled nested probes and autoradiography. Control cultures showed rapid appearance of 2-LTR (arrow), which was partially blocked by rsCD4 and totally blocked by zidovudine and saquinavir. There was little effect of rsCD4 on HIV-1 *gag* (arrow*), while zidovudine decreased *gag* substantially and saquinavir blocked it almost completely. **B:** Effect of zidovudine and saquinavir on accumulation of HIV-1 2-LTR and *gag* DNA in PBMCs.

HIV-1 p24 antigen represented production of defective, noninfectious virions from initially infected cells [Craig et al., 1991].

It has been shown both in vivo [Zhang et al., 1994] and in vitro [Lori et al., 1992] that there exists partially reverse-transcribed HIV DNA packaged within infectious HIV-1 virions. The concentrations of zidovudine we used may have allowed some reverse transcription of HIV-1 RNA within the assembling virions and thus partially transcribed HIV-1 DNA may have been present in infecting virus particles. This could have contributed to the presence of low levels of HIV-1 *gag* DNA observed in the zidovudine-treated cultures. In contrast, saquinavir may have inhibited more effectively the packaging of HIV-1 DNA partial transcripts and blocked the packaging of the virion RNA and processing of structural proteins into infectious virions.

Acute HIV-1 infection of CD4$^+$ cells in vitro demonstrates accumulation of ~80 copies of unintegrated, incomplete sequences of HIV-1 proviral DNA per cell within 48 hr of infection [Robinson and Zinkus, 1990]. It has been shown that the dominant species of HIV-1 DNA in vivo is a full-length unintegrated linear moiety with less than 10% as unintegrated 1- and 2-LTR circular DNA [Chun et al., 1997a]. We showed that unintegrated 2-LTR circular HIV-1 DNA appears in stimulated ACH-2 cells, suggesting that unintegrated HIV-1 DNA in chronically infected cell lines probably represents reinfection. Thus, our data suggest but do not prove that the accumulation of unintegrated 2-LTR circular DNA results from de novo cell infection or reinfection. As such, 2-LTR circular DNA may be a more sensitive indicator of antiviral effect compared to measures of HIV-1 p24 antigen or viral genomic RNA. We

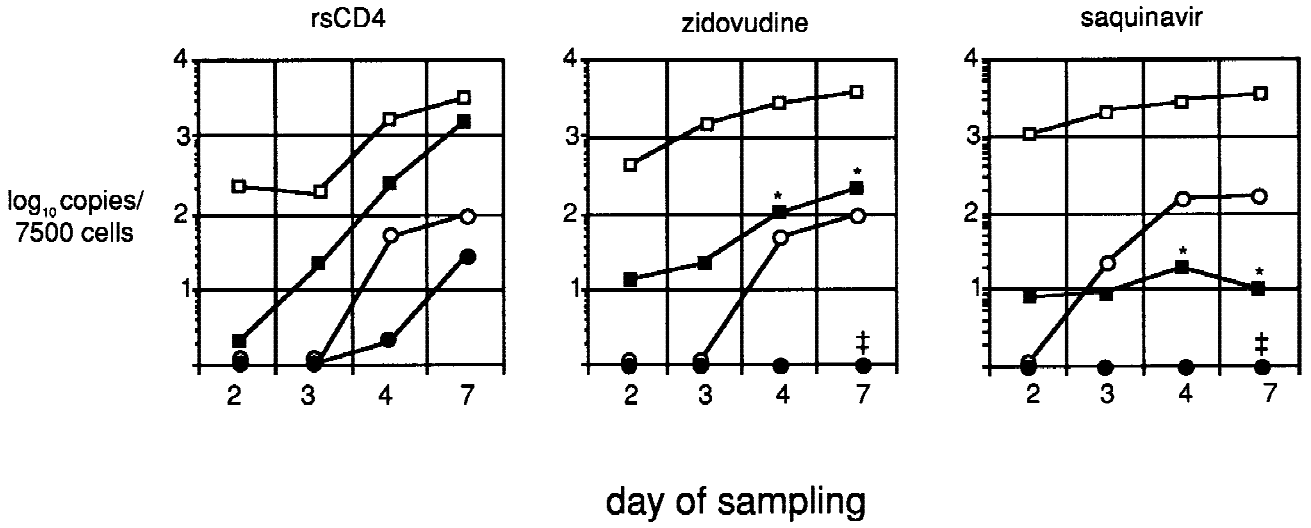


Fig. 4. Summary of semiquantitative PCR from control and drug-treated CEM cultures. Data points indicate mean values. Control *gag* and 2-LTR copies are represented by open boxes and circles, respectively. Solid boxes represent *gag* levels and solid circles represent 2-LTR levels in drug-treated CEM cultures. In the cultures treated with either zidovudine or saquinavir, HIV-1 *gag* was lower compared to control on days 4 (*$P$ = 0.002, ANOVA) and day 7 (*$P$ < 0.0001, ANOVA). Treatment with either zidovudine or saquinavir also inhibited the appearance of HIV-1 2-LTR by day 7 compared to control (‡$P$ < 0.0001, ANOVA). HIV-1 *gag* and 2-LTR levels were not significantly affected by rsCD4 treatment.

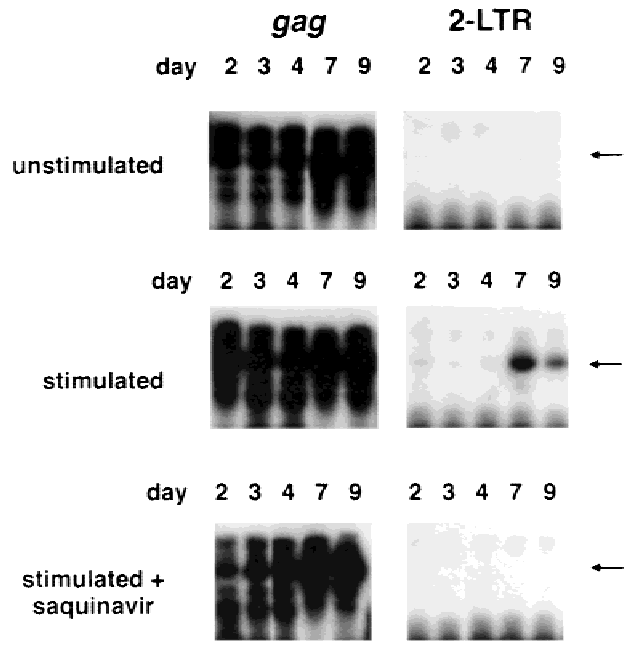


Fig. 5. Appearance of unintegrated 2-LTR HIV-1 DNA after phytohemagglutinin (PHA) stimulation of chronically infected ACH-2 cells in the presence or absence of saquinavir. Stimulation of ACH-2 cells resulted in detection of 2-LTR (arrow), which was blocked by addition of saquinavir.

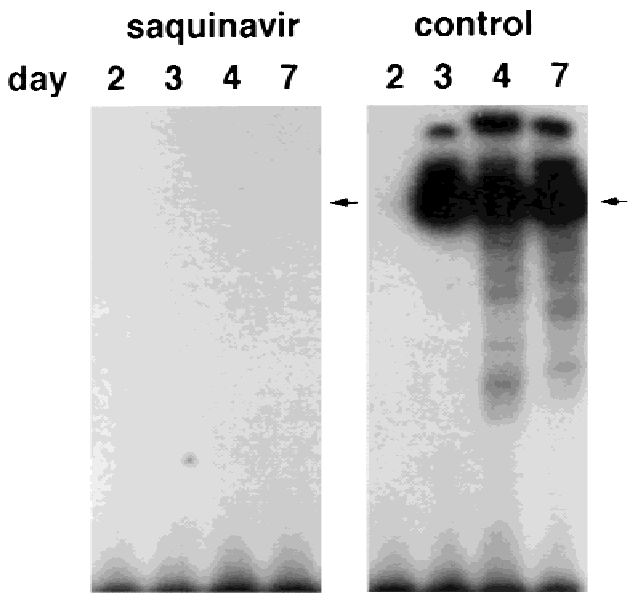


Fig. 6. Effect of saquinavir on appearance of *tat* mRNA in CEM cells acutely infected with HIV-1. Appearance of *tat* mRNA (arrow) was completely blocked in the saquinavir-treated cultures.

also postulate that the 2-LTR circles are a marker of the small portion of virus that is replication competent [Chun et al., 1997a, 1997b]. Further validation of this using clinical specimens is in progress.

The absence of *gag* HIV-1 DNA in the saquinavir-treated CEM cell cultures and the marked delay in its appearance in PMBCs argue for a point of viral interference prior to reverse transcription of genomic viral RNA into proviral DNA. The absence of *tat* mRNA indicates insufficient integrated provirus for gene transcription at our level of assay sensitivity. In agreement with our findings, using an in vitro model for a single cycle of infection, Nagy et al. [1994] and Baboonian et al. [1991] have shown that protease inhibitors UK-88,947 and Ro 31-8959 (saquinavir) were inhibitory at some point between the initiation of reverse transcription and the formation of full-length integrated or un-

integrated forms of HIV-1; however, this has not been confirmed [Jacobsen et al., 1992]. The finding that HIV-1 protease might act at an early step in viral replication is in accord with an in vitro study of equine infectious anemia virus (EIAV) protease [Roberts et al., 1991]. The speculation that protease inhibitors may act at two distinct sites of retroviral replication may be one factor contributing to the enhanced in vivo antiviral potency of these drugs [Collier et al., 1996; Gulick et al., 1997; Hammer et al., 1997].

If the accumulation of unintegrated HIV-1 DNA represents either de novo cell infection or reinfection of already-infected cells, then it may be a plausible biologic marker for active HIV-1 replication and clinical progression [Pauza et al., 1990; Bush et al., 1993, 1996; Donovan et al., 1994a, 1994b; Jurriaans et al., 1995; Nicholson et al., 1996; Zazzi et al., 1997; Panther et al., 1998]. For example, patients with neurological manifestations of HIV-1 have a higher ratio of unintegrated to integrated HIV-1 DNA in their brain tissue than HIV-positive patients without clinical symptoms [Pang et al., 1990; Teo et al., 1997]. In addition, initiation of antiretroviral therapy results in decreased levels of unintegrated HIV-1 DNA in the PBMCs of patients followed longitudinally. This observation suggests that unintegrated viral DNA may be a dynamic clinical marker for active HIV-1 replication and infection or reinfection of $CD4^+$ cells [Dickover et al., 1992; Bush et al., 1993; Donovan et al., 1994a, 1994b; Panther et al., 1998].

In summary, we have shown, as have others, that 2-LTR circular HIV-1 DNA is a quantifiable form of unintegrated HIV proviral DNA in acutely infected PBMCs, CEM cells, and ACH-2 cells. Unintegrated 2-LTR HIV-1 proviral DNA appeared to be a specific marker for de novo cell infection, and possibly reinfection of susceptible cells and measurement of this HIV-1 proviral form may be a useful aid in the study of an antiretroviral drug's mechanism of action.

## ACKNOWLEDGMENTS

We are indebted to Joel Gibson for technical assistance, to Dr. Ian Duncan for supplying saquinavir, and to Nancy Coomer and Pam Easterling for their help in manuscript preparation.

## REFERENCES

Baboonian C, Dagleish A, Bountiff L, Gross J, Oroslan S, Rickett G, Smith-Burchnell C, Troke P, Merson J. 1991. HIV-1 proteinase is required for synthesis of pro-viral DNA. Biochem Biophys Res Comm 179:17–24.

Besansky NJ, Butera ST, Sinha S, Folks TM. 1991. Unintegrated human immunodeficiency virus type 1 DNA in chronically infected cell lines is not correlated with surface CD4 expression. J Virol 65:2695–2698.

Brahic M, Stowring L, Ventura P, Haase AT. 1981. Gene expression in visna virus infection in sheep. Nature 292:240–242.

Bush CE, Donovan RM, Smereck SM, Strang D, Markowitz N, Saravolatz LD. 1993. Quantitation of unintegrated HIV-1 DNA in asymptomatic patients in the presence or absence of antiretroviral therapy. AIDS Res Hum Retroviruses 9:183–187.

Bush CE, Donovan RM, Manzor O, Baxa D, Moore E, Cohen F, Saravolatz LD. 1996. Comparison of HIV type 1 RNA plasma viremia, p24 antigenemia, and unintegrated DNA as viral load markers in pediatric patients. AIDS Res Hum Retroviruses 12:11–15.

Chen ISY, Temin HM. 1982. Establishment of infection by spleen necrosis virus: inhibition in stationary cells and the role of secondary infection. J Virol 41:183–191.

Chowdhury MI, Koyanagi Y, Suzuki M, Kobayashi S, Yamaguchi K, Yamamoto N. 1992. Increased production of human immunodeficiency virus (HIV) in HIV- induced syncytia formation: an efficient infection process. Virus Genes 6:63–78.

Chun TW, Carruth L, Finzi D, Shen X, DiGiuseppe JA, Taylor H, Hermankova M, Chadwick K, Margolick J, Quinn TC, Kuo YH, Brookmeyer R, Zeiger MA, Barditch-Crovo P, Siliciano RF. 1997a. Quantification of latent tissue reservoirs and total body viral load in HIV-1 infection. Nature 387:183–188.

Chun TW, Stuyver L, Mizell SB, Ehler LA, Mican JA, Baseler M, Lloyd AL, Nowak MA, Fauci AS. 1997b. Presence of an inducible HIV-1 latent reservoir during highly active antiretroviral therapy. Proc Nat Acad Sci USA 94:13193–13197.

Collier AC, Coombs RW, Schoenfeld DA, Bassett RL, Timpone J, Baruch A, Jones M, Facey K, Whitacre C, McAuliffe JJ, Friedman HM, Merigan TC, Reichman RC, Hooper C, Corey L. 1996. Treatment of human immunodeficiency virus infection with saquinavir, zidovudine, and zalcitabine. N Engl J Med 334:1011–1017.

Coombs RW, Collier AC, Allain J-P, Nikora B, Leuther M, Gjerset GF, Corey L. 1989. Plasma viremia in human immunodeficiency virus infection. N Engl J Med 321:1626–1631.

Craig JC, Duncan IB, Hockley D, Roberts NA, Mills JS. 1991. Antiviral properties of Ro 31-8959, an inhibitor of human immunodeficiency virus (HIV) proteinase. Antiviral Res 16:295–305.

Dickover RE, Donovan RM, Goldstein E, Cohen SH, Bolton V, Huth RG, Liu GZ, Carlson JR. 1992. Decreases in unintegrated HIV DNA are associated with antiretroviral therapy in AIDS patients. J Acquir Immune Defic Syndr 5:31–36.

Donovan RM, Bush CE, Smereck SM, Baxa DM, Markowitz NP, Saravolatz LD. 1994a. Rapid decrease in unintegrated human immunodeficiency virus DNA after the initiation of nucleoside therapy. J Inf Dis 170:202–205.

Donovan RM, Bush CE, Smereck SM, Moore E, Cohen F, Saravolatz LD. 1994b. Antiretroviral therapy is associated with a decrease in unintegrated HIV-1 DNA in pediatric patients. J Acquir Immune Defic Syndr 7:1237–1241.

Farnet CM, Haseltine WA. 1991. Circularization of human immunodeficiency virus type 1 DNA in vitro. J Virol 65:6942–6952.

Foley GE, Lazarus H, Farber S, Uzman BG, Boone BA, McCarthy RE. 1965. Continuous culture of human lymphoblasts from peripheral blood of a child with acute leukemia. Cancer 4:522–529.

Furtado MR, Balachandran R, Gupta P, Wolinsky SM. 1991. Analysis of alternatively spliced human immunodeficiency virus type-1 mRNA species, one of which encodes a novel *tat-env* fusion protein. Virology 185:258–270.

Gomatos PJ, Stamatos NM, Gendelman HE, Fowler A, Hoover DL, Kalter DC, Burke DS, Tramont EC, Meltzer MS. 1990. Relative inefficiency of soluble recombinant CD4 for inhibiton of infection by monocyte-tropic HIV in monocytes and T-cells. J Immunol 144: 4183–4188.

Guan M, Tudor G, Yang JY, Henderson EE. 1997. Structure and origin of HIV type 1 DNA in persistently infected B lymphoblastoid cell lines. AIDS Res Hum Retroviruses 13:751–757.

Gulick RM, Mellors JW, Havlir D, Eron JJ, Gonzalez C, McMahon D, Richman DD, Valentine FT, Jonas L, Meibohm A, Emini EA, Chodakewitz JA. 1997. Treatment with indinavir, zidovudine, and lamivudine in adults with human immunodeficiency virus infection and prior antiretroviral therapy. N Engl J Med 337:734–739.

Hammer SM, Squires KE, Hughes MD, Grimes JM, Demeter LM, Currier JS, Eron JJ, Feinberg JE, Balfour HH Jr, Deyton LR, Chodakewitz JA, Fischl MA. 1997. A controlled trial of two nucleoside analogues plus indinavir in persons with human immunodeficiency virus infection and CD4 counts of 200 per cubic millimeter or less. N Engl J Med 337:725–733.

Jacobsen H, Ahlborn-Laake L, Gugel R, Mous J. 1992. Progression of early steps of human immunodeficiency virus type 1 replication in the presence of an inhibitor of viral protease. J Virol 66:5087–5091.

Jurriaans S, de Ronde A, Dekker J, Cornelissen M, Goudsmit J. 1995. Increased number of single-LTR HIV-1 DNA junctions correlates with HIV-1 antigen expression and $CD4^+$ cell decline in vivo. J Med Virol 45:91–98.

Kawamura I, Koga Y, Oh-hori N, Onodera K, Kimura G, Monoto K. 1989. Depletion of the surface CD4 molecule by the envelope protein of human immunodeficiency virus expressed in a human CD4 monocytoid cell line. J Virol 63:3748–3754.

Kulkosky J, Katz RA, Skalka AM. 1990. Terminal nucleotides of the preintegrative linear form of HIV-1 DNA deduced from the sequence of circular DNA junctions. J Acquir Immune Defic Syndr 3:852–858.

Kwok S, Erlich G, Poiesz B, Kalish R, Sninsky JJ. 1988. Enzymatic amplification of HTLV-I viral sequences from peripheral blood mononuclar cells and infected tissues. Blood 72:1117–1123.

Laurent-Crawford AG, Hovanessian AG. 1993. The cytopathic effect of human immunodeficiency virus is independent of high levels of unintegrated viral DNA accumulated in response to superinfection of cells. J Gen Virol 74:2619–2628.

Levy JA, Kaminsky LS, Morrow WJW, Steimer K, Luciw P, Dina D, Hoxie J, Oshiro L. 1985. Infection by the retrovirus associated with the acquired immunodeficiency syndrome. Ann Int Med 103: 694–699.

Lori F, Veronese FdM, de Vico AL, Lusso P, Reitz MS, Gallo RC. 1992. Viral DNA carried by human immunodeficiency virus type 1 virions. J Virol 66:5067–5074.

Marquina S, Libonatti O, Ceballos A, Carrillo MG, Peralta LM, Rabinovich RD. 1997. Different integrated and unintegrated HIV-1 DNA after superinfection and cell to cell transmission. Acta Physiol Pharmacol Ther Latinoam 47:245–250.

Masquelier B, Combeau T, Poveda J-D, Delord B, Pellegrin J-L, Sallafranque-Andreola M-L, Tarrago-Litvak L, Fleury HJA. 1991. In vitro assays show a dissociation of reverse transcriptase activity and core antigen (p24) production in two HIV-1 isolates from a patient receiving long-term treatment with zidovudine (ZDV). J AIDS 4:499–505.

Mullins JI, Dhen CS, Hoover EA. 1986. Disease-specific and tissue-specific production of unintegrated feline leukaemia virus variant DNA in feline AIDS. Nature 319:333–336.

Nagy K, Young M, Baboonian C, Merson J, Whittle P, Oroszlan S. 1994. Antiviral activity of human immunodeficiency virus type 1 protease inhibitors in a single cycle of infection: evidence for a role of protease in the early phase. J Virol 68:757–765.

Nicholson WJ, Shepherd AJ, Aw DW. 1996. Detection of unintegrated HIV type 1 DNA in cell culture and clinical peripheral blood mononuclear cell samples: correlation to disease stage. AIDS Res Hum Retroviruses 12:315–323.

Ou C-Y, Kwok S, Mitchell SW, Mack DH, Sninsky JJ, Krebs JW, Feorino P, Warfield D, Schochetman G. 1988. DNA amplification for direct detection of HIV-1 in DNA of peripheral blood mononuclear cells. Science 230:295–297.

Pang S, Koyanagi Y, Miles S, Wiley C, Vinters HV, Chen IS. 1990. High levels of unintegrated HIV-1 DNA in brain tissue of AIDS dementia patients. Nature 343:85–89.

Panther LA, Coombs RW, Zeh JE, Collier AC, Corey L. 1998. Unintegrated circular HIV-1 DNA in the peripheral mononuclear cells of HIV-1-infected subjects: association with high levels of plasma HIV-1 RNA, rapid decline in CD4 count, and clinical progression to AIDS. J Acquir Immune Defic Syndr Hum Retrovirol 17:303–313.

Pauza CD, Galindo JE, Richman DD. 1990. Reinfection results in accumulation of unintegrated viral DNA in cytopathic and persistent human immunodeficiency virus type 1 infection of CEM cells. J Exp Med 172:1035–1042.

Pauza CD, Trivedi P, McKechnie TS, Richman DD, Graziano FM. 1994. 2-LTR circular viral DNA as a marker for human immunodeficiency virus type 1 infection in vivo. Virology 205:470–478.

Rasty S, Dhruva BR, Schiltz RL, Shih DS, Issel CJ, Montelaro RC. 1990. Proviral DNA integratiion and transcriptional patterns of equine infectious anemia virus during persistent and cytopathic infections. J Virol 64:86–95.

Roberts MM, Copeland TD, Oroslan S. 1991. In situ processing of a retroviral nucleocapsid protein by the viral proteinase. Protein Engineer 4:695–700.

Robinson HL, Zinkus DM. 1990. Accumulation of human immunodeficiency virus type 1 DNA in T cells: results of multiple infection events. J Virol 64:4836–4841.

Sato H, Orenstein J, Dimitrov D, Martin M. 1992. Cell-to-cell spread of HIV-1 occurs within minutes and may not involve the participation of virus particles. Virology 186:712–724.

Shaw GM, Harper ME, Hahn BH, Epstein LJ, Gajdusek DC, Price RW, Savia BA, Petito CK, O'Hara CJ, Groopman JE, Cho E-S, Oleske JM, Wong-Staal F, Gallo RC. 1985. HTLV-III infection in brains of children and adults and AIDS encephalopathy. Science 227:177–181.

Smith JS, Kim SY, Roth MJ. 1990. Analysis of long terminal repeat circle junctions of human immunodeficiency virus type 1. J Virol 64:6286–6290.

Sonza S, Kiernan RE, Maerz AL, Deacon NJ, McPhee DA, Crowe SM. 1994. Accumulation of unintegrated circular viral DNA in monocytes and growth-arrested T cells following infection with HIV-1. J Leukoc Biol 56:289–293.

Stevenson M, Meier C, Mann AM, Chapman N, Wasiak A. 1988. Envelope glycoprotein of HIV induces interference and cytolysis resistance in CD4 cells: mechanism for persistence in AIDS. Cell 53:483–496.

Temin HM, Keshet E, Weller SK. 1980. Correlation of transient accumulation of linear unintegrated viral DNA and transcient cell killing by avian leukosis and reticuloenodotheliosis viruses. Cold Spring Harbor Symp Quant Biol 44:773–778.

Temin HM. 1988. Mechanisms of cell killing/cytopathic effects by nonhuman retroviruses. Rev Inf Dis 10:399–405.

Teo I, Veryard C, Barnes H, An SF, Jones M, Lantos PL, Luthert P, Shaunak S. 1997. Circular forms of unintegrated human immunodeficiency virus type 1 DNA and high levels of viral protein expression: association with dementia and multinucleated giant cells in the brains of patients with AIDS. J Virol 71:2928–2933.

Weller SK, Temin HM. 1981. Cell killing by avian leukosis viruses. J Virol 39:713–721.

Whitcomb JM, Kumar R, Hughes SH. 1990. Sequence of the circle junction of human immunodeficiency virus type 1: implications for reverse transcription and integration. J Virol 64:4903–4906.

Zazzi M, Romano L, Catucci M, Venturi G, De Milito A, Almi P, Gonnelli A, Rubino M, Occhini U, Valensin PE. 1997. Evaluation of the presence of 2-LTR HIV-1 unintegrated DNA as a simple molecular predictor of disease progression. J Med Virol 52:20–25.

Zhang H, Bagasra O, Niikura M, Poiesz BJ, Pomerantz RJ. 1994. Intravirion reverse transcripts in the peripheral blood plasma of human immunodeficiency virus type 1-infected individuals. J Virol 68:7591–7597.